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Cannabinoid receptor 2 undergoes Rab5-mediated internalization and recycles via a Rab11-dependent pathway

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ABSTRACT

Cannabinoid receptor 2 (CB2) is a GPCR highly expressed on the surface of cells of the immune system, supporting its role in immunomodulation. This study has investigated the trafficking properties of this receptor when stably expressed by HEK-293 cells. As previously reported, cell surface CB2 rapidly internalized upon exposure to agonist. Direct evidence of CB2 recycling was observed upon competitive removal of the stimulating agonist by inverse agonist. CB2 also underwent slow constitutive internalization when agonist was absent and was up-regulated in the presence of inverse agonist. Co-expression of CB2 and dominant negative Rab5 resulted in a significantly reduced capacity for receptors to internalize with no effect on recycling of the internalized receptors. Conversely, co-expression with dominant negative Rab11 did not alter the ability of CB2 to internalize but did impair their ability to return to the cell surface. Co-expression of wild-type, dominant negative or constitutively active Rab4 with CB2 did not alter basal surface expression, extent of internalization, or extent of recycling. These results suggest that Rab5 is involved in CB2 endocytosis and that internalized receptors are recycled via a Rab11 associated pathway rather than the rapid Rab4 associated pathway. This report provides the first comprehensive description of CB2 internalization and recycling to date.

1. Introduction

The intense interest in cannabinoid receptor 2 (CB2) as a target for the treatment of inflammatory conditions, neuropathic pain and a variety of other pathologies, coupled with an increase in the range and availability of cannabinoid receptor agonists and antagonists [1], suggests that CB2-specific ligands are likely to progress from animal studies to clinical trial in the near future. Intracellular trafficking pathways control the subcellular distribution of receptors and thereby are fundamental contributors to the ability of receptor ligands to generate effective responses. Improved understanding of CB2 trafficking will therefore provide insight into the responses generated in vitro and in vivo and may aid in formulation of dosing regimes or lead to the identification of novel drug targets with potential to modulate CB2 function indirectly.

Agonist binding induces conformational changes in G-protein coupled receptors (GPCRs), promoting coupling to intracellular proteins and initiating signaling cascades. This also leads to desensitization and internalization of activated receptors as adaptive features, disabling cellular responses to continued agonist exposure. Current studies detailing the trafficking properties of CB2 are limited but clearly indicate that CB2 undergoes phosphorylation [2,3] and internalization following agonist stimulation [2,4,5] or exposure to UVB radiation [6]. The observation that CB2 is repeatedly phosphorylated and dephosphorylated by alternate stimulation with agonist and inverse agonist indicates that CB2 recycling may occur [2], but direct evidence to support this hypothesis is lacking. While these prior studies have demonstrated that agonist-induced internalization occurs, the mechanisms controlling internalization have not yet been identified, nor has the post-endocytic fate of CB2 been investigated directly.

The Rab proteins are the largest family belonging to the Ras superfamily of small GTPases and are involved in numerous trafficking processes throughout the cell [7]. They are tethered to membranes by lipid groups attached to their C-termini and bind GTP in their active state. Hydrolysis of GTP to GDP converts the Rab to an inactive form. Rabs predominantly exist in an active state (GTP-bound) as GDP is rapidly replaced by GTP which is at a high cytosolic concentration [7]. Some of the key Rabs that have been identified to facilitate the transport of membrane associated receptors are Rab4, 5, and 11. Specifically, Rab5

Abbreviations: CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate buffered saline; WT, wild-type

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is associated with internalization of clathrin coated pits and fusion with early endosomes [8,9]; it is therefore located on the cytoplasmic surfaces of the plasma membrane, clathrin coated pits and vesicles, and early endosomes. Rab5 has been demonstrated to be involved in the endocytosis of cannabinoid receptor 1 (CB1) [10] as well as several other GPCRs [7]. CB1 has also been suggested to associate with Rab4 [10] which is generally considered to facilitate rapid recycling of receptors directly from the early endosomes back to the plasma membrane [11], although the ability of CB1 to recycle has been questioned recently [12]. Rab11 was not associated with CB1 trafficking [10] but has been indicated to be involved in the slow recycling of GPCRs via the perinuclear recycling compartment [13]. This study has examined the fate of internalized CB2 and its interactions with Rab proteins with a view to understanding the regulation of CB2 cell surface expression.

2. Materials and methods

All reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated.

2.1. HEK-293 cell line generation and maintenance

HEK-293 cells were purchased from the ATCC (#CRL-1573) and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. A pcDNA3.1 vector encoding human CB2 with three hemeagglutinin (HA) tags at the receptor N-terminus was purchased from the Missouri S&T cDNA Resource Center (www.cdna.org; #CNR020TN00) and the 3xHA CB2 sequence was subcloned using restriction enzymes KpnI (Roche, Mannheim, Germany) and PmeI (New England Biolabs, MA, USA) into the pEF4-V5-HisA vector. This vector was stably transfected into HEK-293 cells with Lipofectamine™ 2000 according to the manufacturer's instructions. Following antibiotic selection, a mixed population of cells expressing 3xHA hCB2 was isolated by flow cytometry. Briefly, cells were dislodged with Versene and labeled with mouse-anti-HA.11 antibody (anti-HA.11; MMS-101P, Covance, Berkeley, CA, USA). Cells were then pelleted by spinning at 500×*g* and resuspended in goat-anti-mouse Alexa 488 fluorescent secondary antibody (Molecular Probes, Eugene, OR, USA) in phenol red free DMEM on ice. After washing twice in PBS cells were resuspended in phenol red-free DMEM and passed through a 24-gauge needle to ensure a single suspension before passing through a Becton Dickinson FACS Vantage™ (BD Biosciences, Franklin Lakes, NJ). Using CellQuest Pro software (BD, Franklin Lakes, NJ) a pool of cells with moderate cell surface fluorescence was gated for and collected. The resulting mixed culture is referred to as HEK-CB2 from this point. This cell line was maintained in the presence of 250 μg/mL Zeocin™.

2.2. Trafficking experiments

HEK-CB2 cells were seeded at 26,000–28,000 cells/well in poly-L-lysine (Sigma-Aldrich, St. Louis, MO) treated 96-well plates (Thermo Fisher Scientific, Nunc GmBH, MA, USA) 18–36 hours prior to stimulation. Experiments with HEK-CB2 cells were performed in DMEM with 5 mg/mL bovine serum albumin (ICPbio, Auckland, New Zealand) (basal media) and stimulations took place at 37 °C with 5% CO_2. Tubes and dispensing vessels for cannabinoid and vehicle containing media were silanized and rinsed with deionized water prior to use (Coatasil; Ajax Finechem, Sydney, NSW, Australia).

Prior to cannabinoid stimulation cells were equilibrated in basal media for 30 minutes and then incubated with anti-HA.11 at 1:500 dilution in basal media for 30 minutes. Following two brief washes with warm basal media cells were stimulated with indicated drugs (HU-308, gifted by Professor Raphael Mechoulam, Hebrew University, Jerusalem; AM630, Tocris Bioscience, Bristol, UK). Between stimulations cells were washed twice with basal media. After the appropriate stimulation time plates were placed on ice for 90 seconds to rapidly cool the cells and halt membrane trafficking. Cells were then incubated with Alexa 488 goat–anti-mouse fluorescent secondary antibody at 1:250 in basal media for 30 minutes at room temperature. Subsequently wells were washed twice with basal media, fixed in 4% paraformaldehyde for 10 minutes at room temperature, and washed with phosphate buffered saline (PBS).

For some experiments a second fluorescent goat-anti-mouse secondary antibody (Alexa 594, Molecular Probes) was incubated with fixed and permeabilized cells to detect anti-HA.11 antibody that was located intracellularly, as well as on the cell surface (total starting cell surface receptor). This antibody was diluted to 1:400 in PBS with 0.2% Triton® X-100, 1% normal goat serum and 0.4 mg/mL thiomersal (Merck, Darmstadt, Germany), and incubated at room temperature for 3 hours followed by three washes in PBS with 0.2% Triton® x-100. Cell nuclei were labeled with Hoechst 33258 (Sigma-Aldrich).

2.3. Transient transfection with Rab expressing vectors

Some experiments required transient transfection of HEK-CB2 cells with vectors prior to stimulation. For these, cells were seeded directly into a transfection mixture containing 2.5 μg DNA and 10 μl Lipofectamine™ 2000 per ml Opti-MEM®, prepared according to the manufacturer's instructions. 96-well plates were pre-treated with poly-L-lysine and 50 μl/well transfection mix added to wells before HEK-CB2 cells were seeded at 45,000 cells/well. After 48 hours, cells were stimulated, fixed, imaged and analyzed as described below.

The vectors containing N-terminally EGFP tagged Rab sequences for Rab4b, 5a or 11a were kindly gifted by Professor Robert Lodge of the Univeristé Laval, Canada. Three variations of each EGFP tagged Rab were used. Wild-type Rabs (Rab WT) contained no mutations. Dominant positive Rabs (Rab GTP) contained a single mutation (Q67L in Rab4, Q79L in Rab5, and Q70L in Rab11), conferring an inability to hydrolyse GTP thereby being constitutively active [14]. Dominant negative Rabs (Rab GDP) contained a single mutation (S22N in Rab4, S34N in Rab5, and S25N in Rab11), causing the resulting protein to be constitutively inactive, preferentially binding GDP [14].

2.4. Image acquisition and analysis

Image acquisition and analysis was based on previously described methods [15]. Briefly, images were acquired with a Discovery-1™ automated fluorescence microscope (Molecular Devices, Sunnyvale, CA) using DAPI (350Ex/465Em), FITC (470Ex/535Em), and TRED (560Ex/650Em) filter sets. Grayscale images were captured at 100× magnification from four sites per well with at least four replicate wells for each stimulation condition. Images that were not focused correctly or included large fluorescent debris were excluded from data analysis. Metamorph® software (v. 6.2r6, Molecular Devices) was utilized for image analysis. The total gray value per cell was calculated by determining total gray value above background for each image and dividing it by the number of cells present. The number of cells was determined using the "Count Nuclei" in-built Metamorph® assay and the total gray value above background was determined using the "Thresholded Average Intensity" journal described by Grimsey et al.[15]. As variation in the intensity of antibody staining and therefore fluorescent signal exists between experiments, normalization to unstimulated controls was necessary to combine results from repeated stimulations. GraphPad Prism (v. 4.02, GraphPad Software) was utilized to generate graphs, fit appropriate models and perform statistical tests. Comparisons were performed using a one- or two-way ANOVA with Bonferroni post-test when significance was reached.

For analysis of the Rab co-expression experiments, basal CB2 cell surface expression in cells transfected with each of the nine different Rab proteins was normalized to cell surface CB2 in cells transfected with vector containing EGFP alone. To determine the extent of internalization, the level of cell surface receptor remaining after 30 minutes agonist

stimulation was normalized to basal cell surface CB2 in cells expressing the same Rab protein. The relative extent of receptor recycling was determined by normalizing the cell surface levels of receptor after agonist stimulation and subsequent agonist washout to the starting (100%) and post-internalization (set to 0%) surface expression levels. An agonist washout timepoint at which recycling was sub-maximal (20 minutes) was chosen so that both increases and decreases in the extent of recycling could be detected.

3. Results

3.1. CB2 agonist-induced internalization

Agonist-induced CB2 internalization was both concentration and time dependent (Fig. 1A and B). Corresponding with a reduction in cell surface labeling, CB2 was internalized to diffusely scattered endosomes as well as a distinct perinuclear clustering of receptors (Fig. 3Bii). Agonist-induced loss of cell surface receptor following a 120 minute stimulation with varying concentrations of HU-308 exhibited a classical sigmoidal shaped curve and an EC50 value of 14.6 ± 5.3 nM (Fig. 1A) which is comparable with published potency values for inhibition of cAMP [16]. At the highest concentrations of agonist used and time points greater than 30 minutes using 1 μM HU-308 a plateau was reached with 30–40% of receptors remaining on the cell surface.

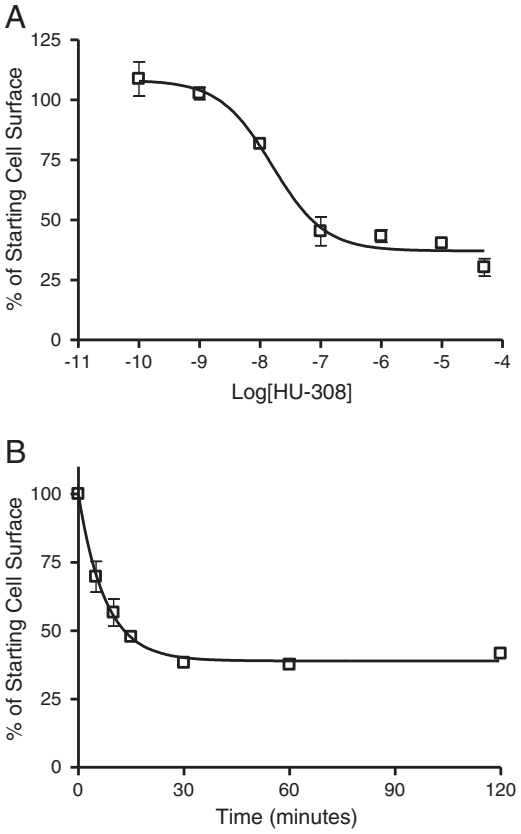

Fig. 1. Internalization of CB2 in HEK-CB2 cells. (A) Concentration dependence of CB2 receptor loss from the cell surface upon endocytosis induced with CB2-specific agonist HU-308 at varying concentrations for 120 minutes, $n = 4$. (B) Receptor loss from the cell surface induced by 1 μM HU-308 with respect to time, $n = 3$. Data was normalized to the cell surface signal of vehicle-treated cells and is presented as means ± SEM of the indicated number of independent experiments (n).

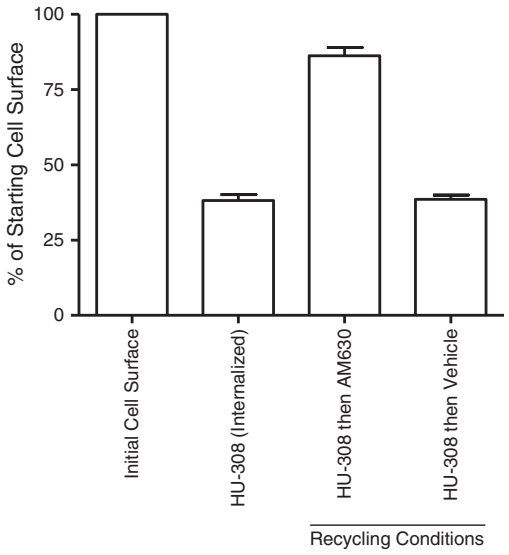

Fig. 2. Agonist washout conditions for CB2 recycling in HEK-CB2 cells. Surface receptor was labeled with primary antibody, then stimulated for 30 minutes with 1 μM HU-308 ("Internalized"). Cells were then washed three times and incubated with AM630 (10 μM) or vehicle for 60 minutes. Secondary antibody was applied under non-permeabilizing conditions to detect antibody-labeled receptor on the cell surface at the end of the drug stimulation. Recycling was observed following agonist-induced internalization when media for the final 60 minute incubation contained AM630, but not with vehicle only. Data was normalized to the cell surface CB2 expression level of unstimulated cells and is presented as means ± SEM of three independent experiments.

3.2. CB2 recycling

Cannabinoid ligands are highly lipophilic and likely to permeate lipid membranes. Thus, removing media after HU-308 stimulation may leave behind lipid solubilized ligand at concentrations high enough to continue to internalize receptors. Indeed, washing with basal media alone was not sufficient to allow for CB2 recycling and the addition of inverse agonist, AM630, was necessary to enable recycling to be visualized (Fig. 2). After internalizing CB2 for 30 minutes with 1 μM HU-308, washing, and applying AM630, the cell surface was replenished to approximately 80% of basal cell surface expression with a half-time of 7.8 ± 3.4 minutes (one-phase exponential association from 0 minutes; Fig. 3). When secondary antibody was applied under permeabilizing conditions, no reduction in the total immunofluorescence was observed ($p = 0.2273$; Fig. 3), indicating that internalized CB2 was not degraded during the time course studied.

Chronic agonist stimulation has been reported to induce a switch from recycling to degradative pathways for some GPCRs [17–19]. We found that after chronic agonist stimulation (4 hours) CB2 was still able to recycle with no evidence of degradation of internalized receptors (Fig. 4). The role of the proportion of receptors that apparently remains sequestered in the cytoplasm following internalization is not yet clear and requires additional investigation.

3.3. CB2 constitutive trafficking

CB2 also underwent internalization when agonist was absent, however, at a much slower rate. Over the time course studied, the process resembled a linear function and receptors were endocytosed at a rate of 5.8 ± 0.2% per hour (Fig. 5A). Constitutively endocytosed CB2 appeared to be degraded, as demonstrated by a reduction in the primary antibody-tagged receptor present in the whole cell over time (Fig. 5A).

Given this constitutive internalization, application of an inverse agonist would be expected to upregulate surface CB2 by stabilizing receptor in an inactive state and thereby preventing endocytosis.

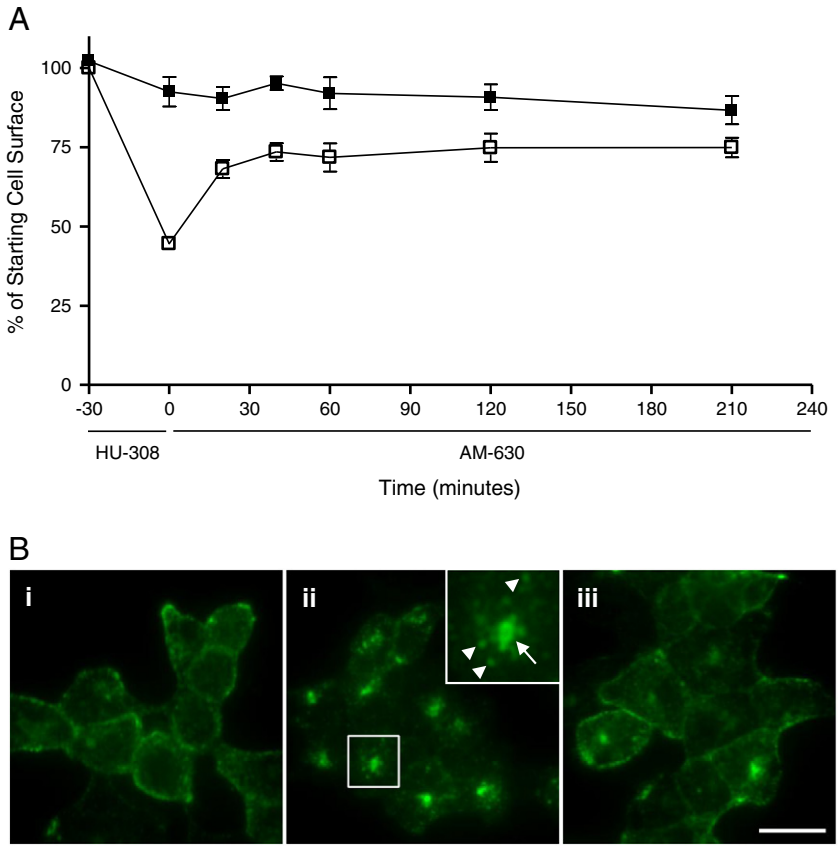

Fig. 3. Recycling of anti-HA labeled CB2 in HEK-CB2 cells. (A) Surface receptor was labeled with primary antibody, then internalized for 30 minutes with 1 μM HU-308. Cells were rapidly washed three times and incubated with AM630 (10 μM) for the times indicated. Secondary antibody was applied under non-permeabilizing conditions to detect antibody-labeled receptor on the cell surface at the end of the drug stimulation (demonstrating CB2 recycling following agonist-induced internalization, □), or under permeabilizing conditions to detect total primary antibody-labeled receptor (demonstrating a lack of CB2 degradation following internalization, ■). Data was normalized to the expression level of unstimulated cells and is presented as the means ± SEM of three independent experiments. (B) (i) HEK-CB2 cells were live labeled with anti-HA.11 antibody and not stimulated, (ii) incubated with 1 μM HU-308 for 30 minutes to induce internalization, or (iii) incubated with HU-308 1 μM for 30 minutes followed by a 60 minute incubation with AM630 (10 μM) to induce recycling. All cells were fixed and permeabilized for detection of intracellular as well as cell surface CB2. Inset details diffusely scattered endosomes (arrowheads) and perinuclear receptor cluster (arrow). Scale bar represents 20 μm.

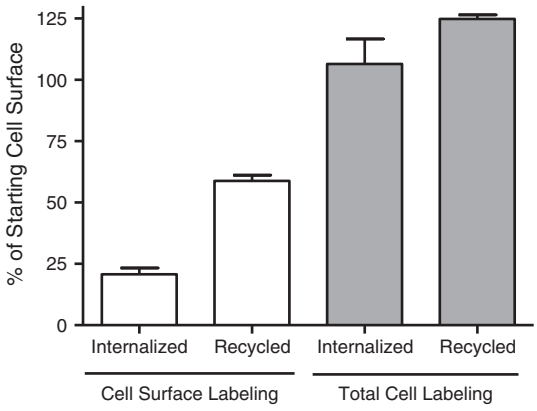

Fig. 4. Effect of chronic HU-308 stimulation on CB2 recycling. Surface receptor was labeled with primary antibody, then stimulated for 4 hours with 1 μM HU-308 ("Internalized"). Cells were washed three times and incubated with AM630 (10 μM) for 60 minutes ("Recycled"). Secondary antibody was applied under non-permeabilizing conditions to detect antibody-labeled receptor on the cell surface at the end of the drug stimulation ("Cell Surface Labeling") or under permeabilizing conditions to detect total primary antibody-labeled receptor ("Total Cell Labeling"). Even following chronic agonist stimulation, CB2 retains a recycling phenotype and is not degraded. Data was normalized to the expression level of unstimulated cells and is presented as the means ± SEM of three independent experiments.

Indeed, incubation with 1 μM AM630 produced a gradual increase in surface CB2 expression ($t_{½}$ 3.3 ± 0.3 hours, one-phase exponential association; Fig. 5B).

3.4. Involvement of Rab GTPases in CB2 trafficking

Investigation of the involvement of Rabs 4, 5 and 11 was performed by transient transfection of each of the EGFP tagged Rab proteins in one of three different states: (1) wild-type (WT), (2) constitutively active (expressing a point mutation that renders the enzyme incapable of hydrolyzing GTP), and (3) dominant negative (the Rabs exhibit a mutation conveying preferential binding to GDP over GTP). Over-expression of the WT Rab proteins did not influence any of the aspects of CB2 trafficking studied, as determined by comparison with cells transfected with empty EGFP vector ($p = 0.9738$).

Transient transfection with Rab5 GDP negatively influenced the extent of agonist-induced internalization ($p = 0.0093$; Fig. 6B) but did not influence the proportion of receptors subsequently recycled ($p = 0.7804$; Fig. 6C). This is consistent with Rab5 being a mediator of CB2 internalization. There was also a trend towards enhanced basal cell surface expression in cells over-expressing this Rab suggesting it may have also impaired constitutive internalization (Fig. 6A).

As expected, the extent of internalization of CB2 was not altered by over-expression of mutant Rab4 or 11 ($p = 0.5687–1$; Fig. 6B). However, the extent of recycling was significantly decreased when Rab11 GDP was over-expressed ($p = 0.0170$; Fig. 6C). There was also a trend

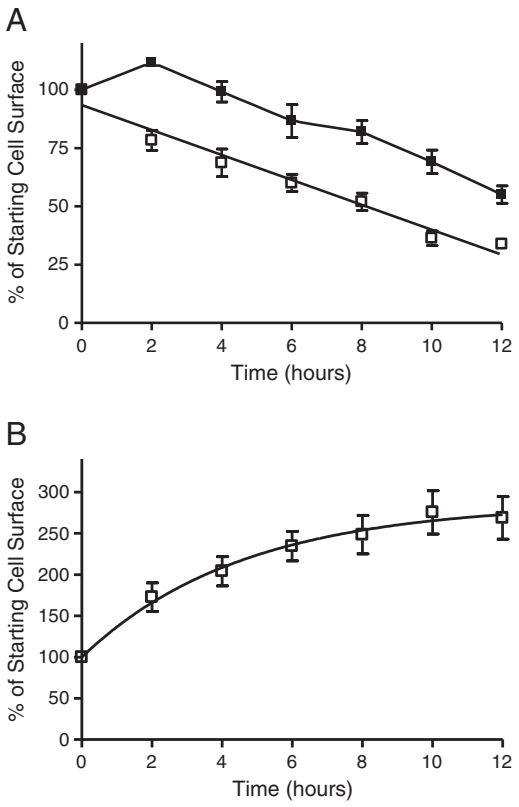

Fig. 5. Constitutive Trafficking of CB2 in HEK-CB2 cells. (A) Surface receptor was labeled with primary antibody, then allowed to constitutively internalize. Secondary antibody was applied under non-permeabilizing conditions to detect antibody-labeled receptor on the cell surface at the end of the incubation (demonstrating constitutive internalization, □), or under permeabilizing conditions to detect total primary antibody-labeled receptor (demonstrating constitutive degradation, ■). (B) Time course of surface CB2 upregulation with 1 μM AM630. Surface receptors were detected by labeling with primary antibody at the conclusion of drug treatment. (A and B) Data was normalized to surface receptor expression level of cells treated with vehicle and fixed immediately following labeling of surface receptors (time 0) and is presented as the means ± SEM of three independent experiments.

towards the constitutively active Rab11 mutant increasing the extent of recycling in comparison with wild-type Rab11, although this did not reach significance ($p = 0.2242$). In contrast, transient transfection with dominant negative (Rab4 GDP) or dominant positive (Rab4 GTP) Rab4 did not significantly alter the extent of recycling ($p = 1$; Fig. 6C). None of the Rab4 or 11 isoforms investigated significantly influenced basal surface CB2 expression ($p = 0.8038$; Fig. 6A).

4. Discussion

Published material describing the trafficking properties of CB2 is scarce but clearly indicates these receptors are capable of undergoing phosphorylation [2,3] and internalization with agonist stimulation [2,4,5] or exposure to UVB rays [6]. CB2 has also been reported to exhibit a degree of constitutive phosphorylation and activation, properties that often correlate with constitutive internalization [2,20]. The present findings support these observations and further characterize the trafficking properties of CB2 in an in vitro, heterologous expression system.

CB2 was rapidly internalized after agonist exposure, as is consistent with other GPCRs [21]. The agonist-induced loss of receptor from the cell surface with respect to time followed a rapid decay curve profile similar to that of CB1 [15]. However in the same parent cell line, close to 100% of CB1 can be induced to internalize [15], whereas here we show the maximum extent of CB2 internalization is approximately 80%. Although it might be suggested that saturation of trafficking pathways was reached in this cell line, similar results were found in various clones of the mixed population exhibiting a range of overall expression levels, as well as in Chinese hamster ovary cells also stably transfected with HA tagged human CB2 (data not shown). This lack of complete internalization is reminiscent of the effects of partial agonists on other GPCRs [e.g., 22–24], and suggests that HU-308 might in fact act as a partial agonist rather than a full agonist as it is sometimes defined [e.g., 25,26].

We observed a significant reduction in the capacity for CB2 internalization in the presence of dominant negative Rab5, clearly demonstrating the involvement of this Rab in CB2 endocytosis. In addition, a trend towards increased basal cell surface CB2 expression when the dominant negative Rab5 was present supports our observation of constitutive CB2 internalization and suggests both agonist driven and constitutive CB2 internalization are mediated by Rab5. The lack of influence of the Rab5 constructs on recycling also indicates that alterations in the extent of internalization do not affect the proportion of CB2 recycled in this model.

A prior report demonstrating the ability of CB2 to be repeatedly phosphorylated and dephophorylated by alternate stimulation with agonist and inverse agonist suggested that CB2 recycling may occur [2]. Here, we provide the first direct evidence to support this hypothesis.

Following internalization we noted that CB2 was localized in both diffuse puncta and a discrete perinuclear cluster in most cells. Interestingly, this pattern resembles a mixture of the phenotypes previously observed for CB1, a degrading receptor exhibiting diffuse endosomes following agonist exposure, and dopamine receptor 1, a receptor that recycles efficiently and is almost exclusively localized in a perinuclear compartment following endocytosis [12]. On this basis it might be deduced that not all endocytosed CB2 is delivered to recycling compartments, and this is indeed consistent with the extent of recycling observed. Unexpectedly, although not all CB2 was recycled to the cell surface, the remaining proportion of internalized CB2 did not degrade and instead seemed to remain sequestered in the cytoplasm. This was also the case following an extended period of agonist simulation. Conversely, CB2 slow degradation was observed following constitutive internalization. While this degradation likely represents cellular turnover of receptors and is not unusual, it is intriguing that agonist stimulation seemed to actively promote recycling and prevent CB2 degradation. The mechanisms and implications of this phenomenon warrant further study.

Our data suggests that CB2 recycling following agonist-induced internalization is mediated via a Rab11-dependent pathway, but does not involve Rab4. While Rab4 appears to be predominantly important for “rapid” recycling directly from early endosomes where receptors have been observed to be reinserted in the plasma membrane within minutes [11,27], Rab11 is generally understood to be required for slower recycling via the perinuclear recycling compartment [13]. Our observation that a proportion of internalized CB2 is localized to a distinct perinuclear cluster is consistent with it being trafficked via the perinuclear recycling compartment and reinforces the conclusion that CB2 undergoes Rab11-dependent recycling.

5. Conclusions

CB2 is under intense scrutiny as a mediator of cannabinoid related effects with the potential to avoid the psychotropic complications involved with activating CB1 receptors. Improved knowledge of the cellular trafficking properties of this receptor may prove beneficial in determining dosing regimes and understanding clinical outcomes. It is apparent from the results obtained here that in transfected cell models CB2 internalizes in response to agonist stimulation, a process involving Rab5 for both constitutive and agonist driven internalization. CB2 recycling following agonist driven internalization involved Rab11 but not Rab4, suggesting recycling occurs through the perinuclear recycling compartment, rather than fast recycling pathway directly from early endosomes. Here we have provided an initial characterization of CB2

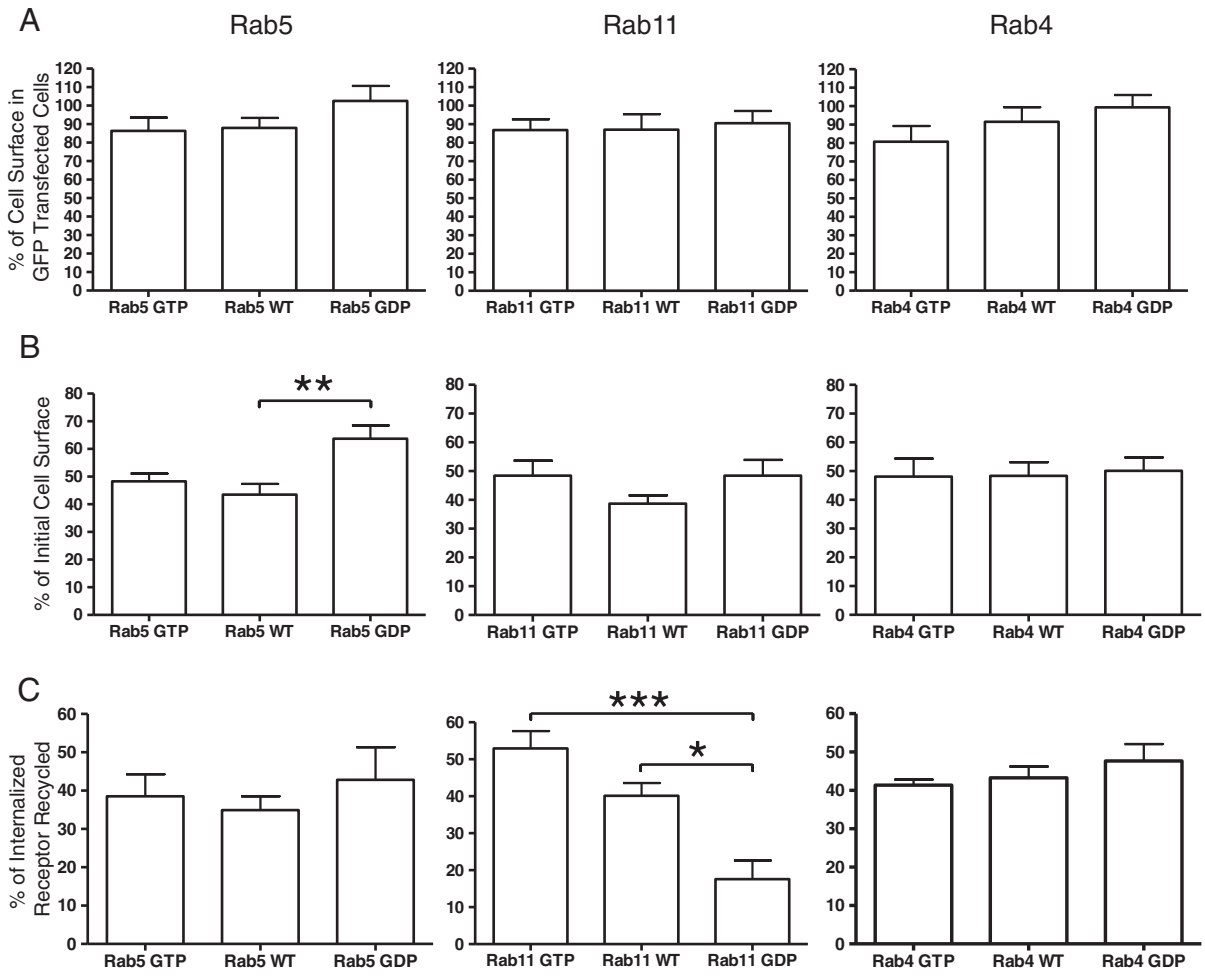

Fig. 6. Effect of Rab over-expression on CB2 trafficking in HEK-CB2 cells. EGFP tagged Rab vectors, as indicated, were transiently transfected into HEK-CB2 cells and data processed as described. (A) Starting surface levels of CB2 relative to that in cells transfected with free EGFP (empty vector). (B) Extent of CB2 internalization induced by incubation with 1 μM HU-308 for 30 minutes. Internalization was inhibited by Rab5 GDP. (C) The relative extent of recycling of CB2 observed following internalization with HU-308 for 30 minutes then incubation with 10 μM AM630 for 60 minutes. Recycling was enhanced by Rab11 GTP and inhibited by Rab11 GDP. Data is presented as the means ± SEM of three to five independent experiments. $^{*}p<0.05$; $^{***}p<0.001$.

recycling in response to the selective agonist HU-308. Future avenues for investigation will be to study trafficking of endogenously expressed CB2 and further define key regulatory proteins involved in this process.

Acknowledgements

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